ISOLATION, PURIFICATION AND CONFORMATIONAL CHARACTERIZATION OF PEANUT (*Arachis hypogea*) LECTIN IN PRESENCE OF CHAOTROPES

Thesis submitted to Department of Life Science for the partial fulfilment of M.Sc. degree in Life Science

DEPARTMENT OF LIFE SCIENCE

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CERTIFICATE

This is to certify that the thesis entitled "Isolation, purification and conformational characterization of peanut (Arachis hypogea) lectin in presence of chaotropes" submitted by K.NANDINI, roll no.413LS2042 for the award of Master of Science degree from National Institute of Technology, Rourkela is a record of bonafide work, carried out by her under my supervision. Results embodied in this thesis serve to be new and has not been submitted to any university for award of any degree or diploma.

10/5/15

DR. SUMAN JHA

DECLARATION

I do hereby declare that the project report entitled "Isolation, purification and conformational characterization of peanut lectin (Arachis hypogea) in presence of chaotropes" submitted to Department of Life Science, National Institute of Technology, Rourkela for the partial fulfillment of Master Degree in Life Science is a faithful record of bonafide and original research work carried out by me under the guidance and supervision of Dr. Suman Jha, Department of Life Science, NIT Rourkela

DATE:

K.NANDINI

PLACE:

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ABBREVIATIONS

°C: Degree Celsius

ConA: Concanavalin A

EDTA: Ethylene Diamine Tetra Acetate

M: Molar

mg: Milligram

mM:Millimolar

ml: Millilitre

OD: Optical Density

PNA: Peanut Agglutinin

SDS PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

µg: Microgram

CD: Circular Dichroism

GdnHCl: Guanidine Hydrochloride

MW: Molecular weight

FT-IR: Fourier Transform Infrared Spectroscopy

$_{P}$H: Power of hydrogen

Fmax: Fluorescence maxima at 470nm

ABSTRACT

Peanut lectin (PNA) is a plant protein isolated and purified from its natural source *Arachis hypogea* using biophysical technique called salting out, and analytical technique called size exclusion chromatography, respectively. The isolated lectin was characterised by SDS-PAGE followed by FT-IR study of peanut lectin, which gives the secondary structure of peanut protein. The FTIR data were further strengthened using Circular Dichroism spectropolarimeter. Although peanut lectin comprises majorly of β-sheet, the protein gave strong negative ellipticity at ~223 nm, a signature of lectin proteins. In addition to circular dichroism study, extrinsic fluorescence study of PNA using 8-Anilino Napthalene-1-Sulphonic acid (ANS) was performed under different conditions such as a range of pH, varying concentration of SDS, and GdnHCl for protein conformational studies. Thermal profiling of PNA was accompanied to study the denaturation pattern of peanut agglutinin, and to know its melting point.

KEYWORDS: Peanut lectin, Plant lectin, Affinity chromatography, SDS-PAGE, 8-Anilino naphthalene-1-sulphonic acid, Guanidine Hydrochloride, FTIR, Circular Dichroism.

1. INTRODUCTION

The peanut or groundnut (*Arachis hypogaea*) is a species in the family fabaceae in general known as the bean pea or legume family. Peanuts are used to combat malnutrition. Lactose devoid of milk like beverage can be prepared using peanut and other grains. Inspite of peanut comprising of mono unsaturated fat content in peanuts show an array of other nutrients that in numerous studies which has been shown to promote heart health. Peanuts seem to be good sources of vitamin E, niacin, folate, protein and manganese. In addition peanuts provide resveratrol, the phenolic antioxidant which is also found in red grapes and red wine(1, 2).

1.1 DAY TO DAY USE OF PEANUT

Peanut oil preparation using low-grade peanuts which are hardly suitable for the edible marketing is in practice. The protein cake (oilcake meal) residue from oil processing is used as an animal feed and as a soil fertilizer. Raw peanuts are also widely sold as a garden birdfeed.

Peanuts have a variety of industrial uses which include production of paint, varnish, lubricating oil, leather dressings, furniture polish; insecticides are made from peanut oil. Soap is made from saponified oil, and many cosmetics also contain peanut oil and its derivatives (2).

1.2 LECTINS

Lectins are glycoproteins of 60,000-100,000 MW that are known for their ability to agglutinate (clump) erythrocytes in vitro.The major function of lectins in animals is to enhance and facilitate cell-cell contact. A lectin usually comprises of two or more binding sites for carbohydrate units; some lectins are found to form oligomeric structures having multiple binding sites. The binding sites of lectins on the surface of one cell interact with number of carbohydrates displayed on the surface of another cell. Lectins and carbohydrates are linked by a number of relatively weak interactions that helps ensure specificity.

Lectins are found to have the potential use in cancer treatment due to the fact that lectins present on the surface of tumour cells are capable of binding exogenous carbohydrate-containing molecules and internalize them by the process of endocytosis(3, 4).

1.2.1 ANTINUITRITIONAL PROPERTIES OF LECTINS

Lectins are carbohydrate binding (glyco) proteins which are found everywhere in nature. They are found in various plants and hence ingested daily in appreciable amounts by both humans and animals. One of the most nutritionally important features of plant lectins is their ability to survive digestion by the gastrointestinal tract of individuals who consume them.

The lectins are found to attach to membrane glycosyl groups of the cells lining the digestive tract. As a result of this interaction a series of harmful local and systemic reactions are triggered placing lectins as antinutritive and /or toxic substances. Locally, they are found to affect the turnover and loss of gut epithelial cells, damage the luminal membranes of the epithelium, interfere with nutrient digestion and absorption, stimulate changes in the bacterial flora and alter the immune state of the digestive tract. Systemically, they can disrupt lipid, carbohydrate and protein metabolism, promote enlargement and/or atrophy of key internal organs and tissues and alter the hormonal and immunological status of consumers. At high intakes, lectins may seriously threaten the growth and health of consuming animals. They are also detrimental to numerous insect pests of crop plants.(4, 5).

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1.3 PEANUT AGGLUTININ (PNA)

Peanut agglutinin is plant lectin protein derived from the fruits of *Arachis hypogaea*. It was the first lectin to be fully studied in this plant.PNA has been reported to be highly specific for the tumor-associated T-antigenic disaccharide Gal (βl-3) GalNAc. Peanut agglutinin has been identified as a tetrameric protein with a molecular weight of 110 KDa. It particularly binds malignant cells; and because of this reason, this lectin has been widely used as a probe for identifying malignant phenotypes in numerous tissues(6).

Peanut agglutinin may also be referred to as *Arachis hypogeae* lectin. The protein is 273 amino acids in length with the first 23 residues acting as a signal peptide..*Arachis hypogaea* lectin or Peanut Agglutinin (PNA) is isolated from peanuts and purified by affinity chromatography. The lectin has a molecular weight of 110 KDa and consists of four identical subunits of MW approximately 28 KDa each(6, 7).

2. REVIEW OF LITERATURE

PNA is a homotetrameric lectin with a molecular weight of 110KD.The subunit conformation in the structure of PNA is similar to that in other legume lectins except when comparing loops. It was shown that in the tertiary structure of legume lectins, the short five-stranded sheet plays a major role in connecting the larger flat six-stranded and curved seven-stranded sheets. Furthermore, the loops that connect the strands at the two ends of the seven-stranded sheet curve toward each other and interact within the sheet to produce a second hydrophobic core in addition to the one between the two large sheets. The ‘‘open’’ quaternary association in peanut lectin is stabilized by hydrophobic, hydrogen-bonded and water-mediated interactions (6, 8, 9).

In other legume lectins, the framework of the molecule consists of three sheets, a six-stranded flat sheet, a seven-stranded curved sheet and a small five-stranded sheet, sheet 3 has a major role in holding the two larger sheets together(8, 10). Loops make up 54% of structure. Loops connect adjacent strands in sheet(8, 10).

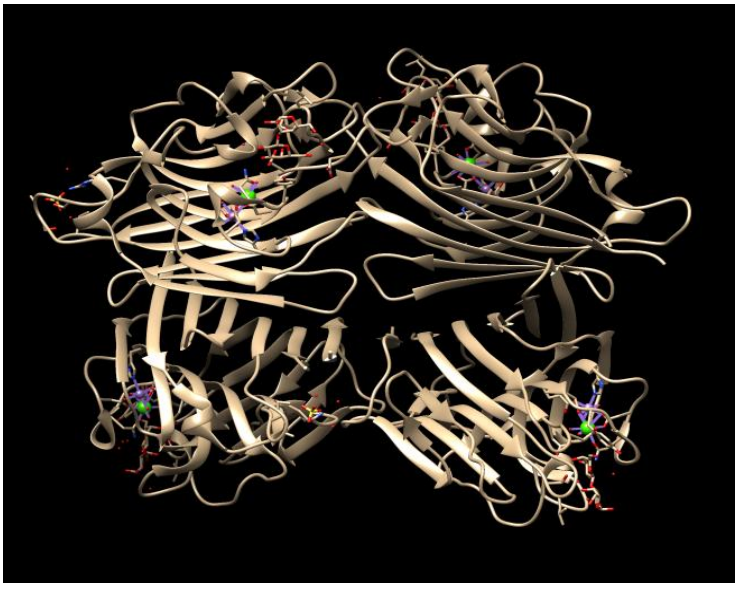

Fig1. Tetrameric structure of PNA: PDB file

(PDB ID : 2DV9, adopted from Natchiar et al. 2006)(11).

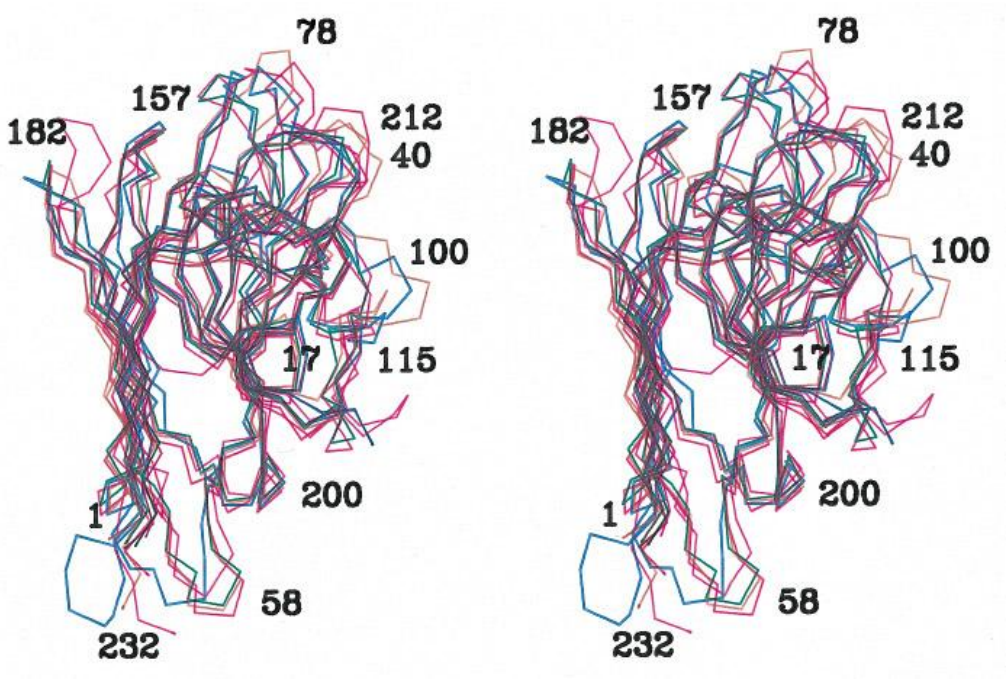

Fig2. Stereo view of the superposition of the subunits of con A (blue), pea lectin (green), EcorL (red) and GS4 (pink) on to PNA subunit 1 (brown). The residue numbering corresponds to the PNA sequence(Adopted from Banerjee et al. 1996)(8).

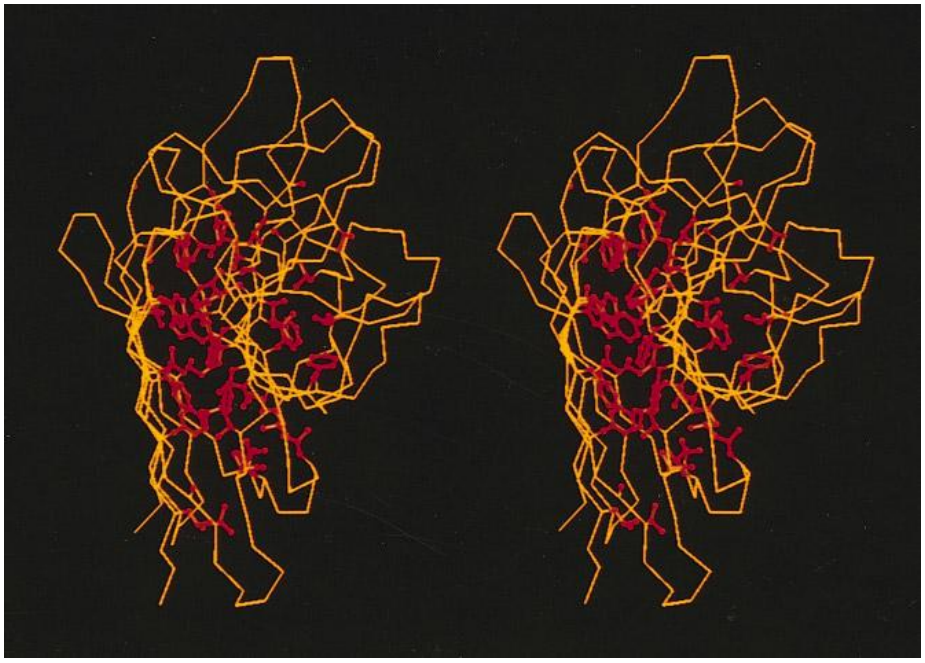

Fig3. View of the buried hydrophobic residues (red) in the subunit with the main-chain shown in yellow (adopted from Vallone et al. 1998)(12).

2.1 METAL BINDING

Each monomer of PNA as in case of other legume lectins contains one calcium ion and one manganese ion. The metal interaction and positioning is almost identical in the four subunits, which are nearly similar to other legume lectins. Although the metal-binding regions are totally conserved in many lectins, the calcium-manganese distances vary between 4.13 and 4.39A° in the four subunits(8).

2.2 QUATERNARY ASSOCIATION IN PNA

One half of the molecule comprises of subunit 1and 4 and stays in association with other half comprising of subunits 2 and 3 by molecular dyad.The tetramer had three subunit interfaces: that between 1 and 2, that between 1 and 4 which is related to that between 2 and 3 by the molecular dyad, and that between 3 and 4 (6).

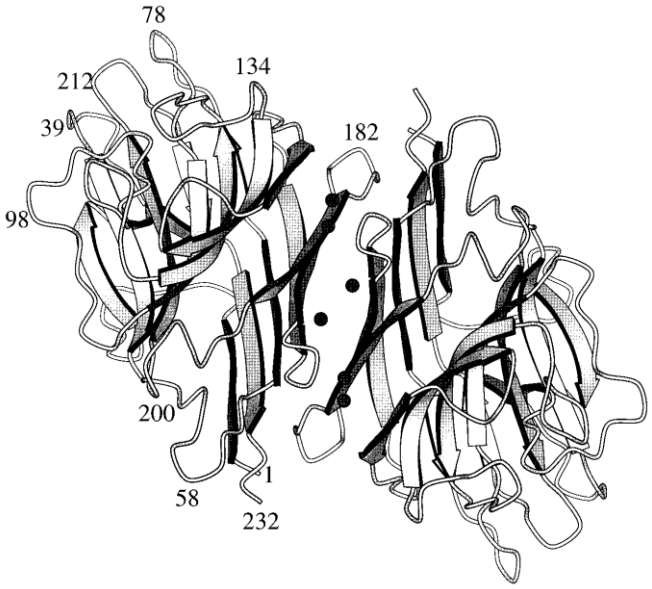

Fig4. The PNA dimer (subunits 1 and 4). The water molecules represented by small filled circles(adopted by Banerjee et al 1996)(10).

The binding region corresponding to the 3-4 interfaces is vacant in subunits 1 and 2 while that corresponding to 1-2 was vacant in subunits 3 and 4. Thus the tetramer was said to have an open structure(6, 8).

2.3 ROLE OF PEANUT AGGLUTININ

Ingested peanut agglutinin profoundly shows stimulation of proliferation of colon in humans. In rats, ingested peanut agglutinin has an impact on hormone release and stimulates proliferation in the small and large intestines. Peanut agglutinin is believed to be absorbed into the circulation but little was known about the systemic effect of peanut lectin. Experiment conducted on rats revealed that intravenous dose of varying concentration of peanut agglutinin stimulated proliferation in the mid colon, the proliferation will lead to colonic carcinogenesis.(13) Peanut agglutinin had no effect on pancreas, enzyme levels or DNA content but varying dose increased plasma concentrations of enteroglucagon and glucagon-like peptide-1which ultimately lead to the proliferation of distal colon in small intestine.(13) Peanut agglutinin might be useful in therapeutics. Stimulation of intestinal proliferation by peanut agglutinin, or other lectins, could be used to counteract the atrophy (complete wasting away of a part of a body) produced by total parenteral nutrition, to aid in the healing of surgical anastomoses, or to promote healing in inflammatory conditions such as colitis. Peanut agglutinin is well tolerated by the animals and no histological changes were evident in the past studies but recent studies say that, peanut agglutinin-stimulate glucagon-like peptide-1 release might be useful in type-2 diabetes. The proliferative effects of peanut agglutinin in the gastrointestinal tract were of considerable effect in causing cancer and were thought to be mediated as a part of circulation.(13)

2.4 TECHNIQUES EMPLOYED FOR THE BIOPHYSICAL CHARACTERIZATION

2.4.1 DIALYSIS

Dialysis is a separation process where ion-exchange membrane (IEM) is used and the process is driven by difference in concentration gradient and the sample is applied for separation and acid/alkali waste solutions are recovered in a cost-effective and environment friendly manner. In Dialysis the solutes moves across the semi-permeable membrane by which solutes dissolve and semi-permeable membrane acts as a source of ultrafilteration of fluids (14). In water substances attains property of diffusion; movement of solute molecules takes place from a region of low concentration to a region of high concentration. Pores of various sizes in a material which is thin layered is called as semi-permeable membrane. Salts and fluids which

are categorized as smaller solutes may pass through membrane, but the passage of large molecules like protein may block the semi-permeable membrane(15).

2.4.2 SIZE-EXCLUSION CHROMATOGRAPHY

Size exclusion chromatography (SEC) is the process where mixtures are separated on the basis of their molecular size, more specifically the hydrodynamic volume of the components. As the solutes pass through the stationary phase there occurs a differential exclusion and inclusion of solutes because the stationary phase consist of pores of different sizes in the gel or beads used. Rate of permeation of different solutes vary inside the particles in gels. Size exclusion chromatography is a process with samples are held gently(16).

2.4.3 SDS-PAGE

Electrophoresis involving proteins serves as a tool to study the properties of proteins. During electrophoresis movement of molecules in a gel will separate proteins on the basis of their molecular size and these charged molecules moves under the effect of electric field. Polyacrylamide gel is often used to separate proteins. Polyacrylamide is formed from chemical agents acrylamide and bisacrylamide. Ammonium persulfate (APS) and TEMED (Tetramethylethylenediamine) are the components which are being used to polymerize gel. In Polyacrylamide gel pores with different sizes are formed by cross linking of acrylamide and bis-acrylamide which depends on the percentage of acrylamide mix being used. More the percentage of acrylamide mix smaller the pores will be. Small molecules migrate rapidly through the pores and the larger molecules migrate slower. Proteins are separated on the basis of their molecular size i.e. mass to charge ratio. Sodium Dodecyl Sulfate (SDS) is an anionic detergent that imparts a uniform negative charge to the protein which results in protein denaturation unfolding of each polypeptide chain into a linear one. An SDS-PAGE is composed of resolving gel and stacking gel. Resolving gel has a pH of 8.8 and stacking gel has a pH 6.8.Resolving gel comprising of anionic chloride and glycine carry most of the current. The proteins present in the sample encounter with high pH and smaller pore size. The increase in pH would tend to increase electrophoretic mobility but, smaller pores decrease mobility. Hence, the relative rate of movement of protein is lesser than chloride and glycine. This process lead to better separation of proteins based on charge/mass ratio and a discrete size and shape. The electrophoretic mobility of the SDS-protein complexes is influenced primarily by molecular size: the larger molecules experience sieving effect, a property displayed by the gel and the smaller molecules tend to show greater mobility(17).

2.4.4 UV-VIS SPECTROPHOTOMETRY

A spectrophotometer is used to measure the amount of light absorbed by the sample. In spectrophotometer a beam of light is passed through the sample and the intensity of light is measured. Sample absorbs light when photons pass through the cuvette. The number of photons in the beam of light gets reduced which in turn reduces the intensity and energy of photon. First, the intensity of light (I_0) passing through a blank is measured. The intensity is the number of photons per second. The blank is the solution identical to the sample solution except that the blank does not contain the solute that absorbs light. Second, the intensity of light (I) passing through the sample solution is measured. Thus, the experimental data is used to calculate two quantities: the transmittance (T) and the absorbance (A) are given by the equation:

$$T = \frac{I}{I_0}$$

$$A = -\log_{10} T$$

The transmittance is the fraction of light in the original beam that passes through the sample and reaches the detector. The remainder of the light, 1 - T, is the fraction of the light absorbed by the sample. If no light is absorbed, the absorbance is zero (i.e. 100% transmittance)(18).

2.4.5 FT-IR

FTIR spectroscopy is a measurement of wavelength and intensity of the absorption of IR radiation by the sample. Every molecule has a property to absorb light in the Infrared region, which causes the bonds within the molecules to stretch and vibrate. Each kind of bond vibrates at a different wavenumber. This property is used to characterize different types of molecules. For proteins, the most sensitive spectral region is the amide I bands (1700-1600 cm^{-1}) which is mainly due to C=O stretching vibrations. A region ranging from 1658 and 1650 cm^{-1} is assigned to α-helix. Bands in the regions of 1640-1620 cm^{-1} and 1695-1690 cm^{-1} are assigned to β-sheet. Random coil is associated with the IR band between 1640 and 1648 cm^{-1} (19).

2.4.6 CIRCULAR DICHROISM

Circular dichroism is very sensitive to secondary structure of polypeptides and proteins. Circular dichroism (CD) spectroscopy is a form of light absorption spectroscopy that

measures the difference in absorbance of right- and left-circularly polarized light. Inherently asymmetric chromophores (uncommon) or symmetric chromophores in asymmetric environments will interact differently with right- and left-circularly polarized light resulting in two related phenomena. Circularly-polarized light rays will travel through an optically active medium with different velocities due to the different indices of refraction for right- and left-circularly polarized light called optical rotation or circular birefringence. The variation of optical rotation as a function of wavelength is called optical rotary dispersion (ORD). Right- and left-circularly polarized light will also be absorbed to different extents at some wavelengths due to differences in extinction coefficients for the two polarized rays called circular dichroism (CD). Circularly polarized light is a form of polarization wherein the magnitude of the oscillation is constant and the direction oscillates. The differential absorption of radiation polarized in two directions as function of frequency is called dichroism. Left and right circularly handed polarized components of the incident light are detected differently by the sample, which yields a difference in absorption coefficient$\Delta\varepsilon = \Delta\varepsilon left - \Delta\varepsilon right.$ This latter difference is called circular dichroism. The chromophore responsible for chiral absorption phenomenon is the peptide bond. The secondary structure of polypeptide gives rise to CD phenomenon of protein in the wavelength interval 290 - 160 nm(20).

2.47 EXTRINSIC FLUORESCENCE SPECTROSCOPY USING ANS

8-Anilinonaphthalene-1-sulfonic acid (ANS) is an organic compound containing both a sulfonic acid and an amine group. This compound is used as an extrinsic fluorescent molecular probe. ANS can be used to study conformational changes induced by ligand binding in proteins, as ANS's fluorescent properties will change as it binds to hydrophobic regions on the protein surface. Comparison of the fluorescence in the presence and absence of a particular ligand can thus give information about how the binding of the ligand changes the surface of the protein(21).

2.5 EFFECT OF pH ON PNA

PH influences the conformation of peanut agglutinin, PNA is found to be tetrameric in neutral solution. A lower pH below 5.1 causes a reversible dimerization of PNA. pH lower than 3.5 is the condition where lectin is totally dimeric. Microenvironment of PNA-bound chromophore change progressively with pH and is dependent on ionization of an acidic

amino acid residue was concluded from fluorescence studies of PNA as a function of pH in the presence of lactose(22).

2.6 EFFECT OF GdnHCl ON PNA

Denaturants like urea and guanidine hydrochloride are strong chaotropic agents have long been associated with studying protein denaturation and conformations(23). Guanidine hydrochloride (GdnHCl) is believed to be an ideal chemical denaturant for protein unfolding reaction. It is generally believed that binding or interaction of GdnHCl occurs to both folded and unfolded states of but the binding affinity and the number of binding sites in each of the states is different. The precise mechanism however is not understood properly(24)Study of unfolding process of PNA, induced by GdnHCl revealed that intermediates during unfolding process is found to have 80% of structural element intact which is found in secondary structure of PNA and it was concluded from the fluorescence study that despite of reduced tertiary structure it retains the carbohydrate binding activity(25).

3. OBJECTIVES OF THE STUDY

1. Isolation of peanut agglutinin from peanut (***Arachis hypogea***) seeds.
2. Conformational characterization and thermal profiling of the isolated lectin.
3. Conformational characterization of the isolated protein in presence of different chaotropes.

4. MATERIALS AND METHODS

Dried peanuts were purchased from local grocery shop in Rourkela, Odisha, India.

4.1 CHEMICALS REQUIRED

Ammonium sulphate salt, GuanidineHCl, 8-Anilino naphthalene-1-sulphonic acid was purchased from HiMedia, Sodium dihydrogen phosphate, hydrogen disodium phosphate Sigma, Sephadex G-100, Sodium dodecyl sulphate was purchased from Sigma, 5X Bradford reagent was purchased from Bio-Rad.

4.2 GLASSWARES AND PLASTIC WARES

Glass column (purchased from Borosil), measuring cylinder, Beaker, test tubes, Eppendorf tubes, tips (Purchased from Tarson) and accessories, Microplate (purchased from Nest).

SDS-PAGE

TABLE 1: 10% RESOLVING GEL COMPOSITION

10% RESOLVING GEL	QUANTITY
30% acrylamide mix	2.67ml
Distilled water	3.15ml
Tris 1.5 M, pH -8.8	2ml
10% SDS	80µl
10% APS (Ammonium per sulphate)	80µl
N,N,N,'N'-Tetraethylmethylenediamine (TEMED)	8µl

TABLE 2: 6% STACKING GEL COMPOSITION

6% STACKING GEL	QUANTITY
30 % acrylamide mix	300µl
Distilled water	1.89ml
TRIS 0.5 M, pH -6.8	750µl
10% SDS	20µl
10% APS	20µl
TEMED	4µl

4. 3 METHODOLOGY

4.3.1 EXTRACTION AND PURIFICATION OF LECTINS

Raw peanuts were rinsed using Milli Q water and soaked in 1L Milli Q water overnight. Peanut seeds were then peeled off and were dried by incubating in hot air oven overnight at

50°C. Peanut seeds were then crushed to paste and about 200g of paste was resuspended in 500 ml of Milli Q water. The mixture was incubated for 1 hour at 37°C and was subjected to centrifugation at 7000 rpm for half an hour at room temperature. Supernatant thus obtained was then used for purification of lectins. Major fraction of protein in supernatant was precipitated by salting out method using ammonium sulphate solution (4.1M). To the final volume received after centrifugation 4.1M ammonium sulphate solution to be added was calculated which was required for 30% saturation (cut off) and incubated for 6 hours in magnetic stirrer at 4ºC.The mixture was subjected to two rounds of centrifugation. To the supernatant obtained, ammonium sulphate solution was added which was required for 30%-60% saturation of protein and was kept for precipitation overnight at 4ºC and centrifugation was carried out. Finally 90% saturation was carried out and the mixture was then subjected to centrifugation. The pellet obtained after 90% cut-off was dissolved in least amount of phosphate buffer (10mM, pH=8) and the solution was set for dialysis to remove salt. The dialysis membrane was activated by boiling it with 0.1% EDTA followed by rinsing with MilliQ water. These membranes were checked for leakage and 90% pellet was set for dialysis against Milli Q water which was changed for every 6 hours of dialysis. Three sets of dialysis were placed with the interval of 6 hours. Dialysis follows the principle of osmosis where ammonium sulphate salt concentration is balanced by movement of solute from a region of high concentration to a region of low concentration. The dialysed sample was then applied to size exclusion chromatography column which was prepared by Sephadex G-100 and equlibirated after with phosphate buffer upto 3 bed volumes. Protein was collected as different fractions of 1 ml each in eppendorf tubes at the rate of 1ml/min.

4.3.2 ELECTROPHORESIS

The purity and molecular weight of lectin was confirmed by SDS-PAGE using 10% concentration of polyacrylamide in resolving gel and 6% polyacrylamide in stacking gel. The molecular weight of Peanut lectin was determined by SDS-PAGE.

4.3.3 PROTEIN ESTIMATION USING BRADFORD ASSAY

PRINCIPLE

Bradford method is based on a blue dye (coomassie brilliant blue G250) that binds to free amino groups in side chains of amino acid, especially Lys and other aromatic amino acids

such as tyrosine, tryptophan and histidine along with peptide bonds and gives a characteristic blue colour. On binding of dye to protein an increased absorption is found at 595 nm(26).

PROCEDURE

The protein concentration of the pure eluents as depicted by SDS-PAGE was determined by Bradford assay.

REAGENTS REQUIRED:

- Bradford reagent (1X)
- BSA of known concentration for standard curve (10µg, 20µg, 40µg, 60µg, 80µg, 100µg).

Different volumes of BSA sample/PNA eluants were added to test tubes to a total volume of 0.1 ml. To the protein solution 5 ml of 1X Bradford was added and incubated for30 mins. Then O.D reading was taken at 595 nm in Cary 100 UV-Vis spectrophotometer purchased from Agilant. The graph was plotted for determining the unknown concentration of protein of interest against standard curve.

4.3.4 BIOPHYSICAL CHARACTERIZATION

4.3.4.1 FTIR

FTIR was carried using instrument purchased from BRUKER, Germany for all elutes and the graphs obtained were processed and plotted to analyze the presence of secondary structures such as β-sheet, anti-parallel β-sheet, α-helix, α-helix with some random coil, and random coil in Peanut Agglutinin (abbreviated as PNA).10mM phosphate was taken as blank.

4.3.4.2 CIRCULAR DICHROISM

The cleaned quartz cuvette was used to measure difference between two absorption values, baseline was measured using phosphate buffer in which the protein was dissolved.
Consequently the 23 elutes were set for measurement one after the other. The raw data thus obtained was processed (190-260 nm) and plotted in Origin Pro. Instrument model used was JASCO- J1500 CD spectrophotometer.

4.3.4.3 UNFOLDING STUDIES

Unfolding of PNA was monitored by CD studies and analysis of extrinsic fluorescence using ANS

i. Varying pH ii. Varying SDS concentration iii. Varying GdnHCl concentration

4.4.1 STUDY OF PROTEIN UNFOLDING USING 1-ANILINO-8-NAPTHALENE SULPHONATE (ANS)

The effect of pH, solvent composition and the polarization of fluorescence may contribute to structural elucidation. A common non-conjugating extrinsic chromophore for proteins is 1-anilino-8-napthalene sulphonate (ANS). ANS concentration to be used is previously optimized which accounted to be 0.1 mM and the concentration of protein sample to be used was 0.05mg/ml for all the ANS studies. In a 96 well plate, phosphate buffer, protein sample and buffers of different PH /SDS concentration/GdnHCl concentration was added respectively. After 30 minutes, ANS was added to a final concentration of 0.1 mM and kept for incubation again for 30 min. After incubation, Reading was taken in a microplate reader (BioTek synergy H1). ANS was excited at 388 nm and emission was taken from 420 nm to 580 nm.

4.4.2 STUDY OF SECONDARY STRUCTURE OF PROTEIN AT DIFFERENT PH, AND VARYING CONCENTRATIONS OF SDS AND GdnHCl USING CIRCULAR DICHROISM.

A protein concentration of 0.1 mg/ml was used for CD experiments. The sample was prepared to carry out CD spectrophotometer (JASCO J1500) by using phosphate buffer of different pH (2, 3, 5, 6, 7, 8, 9, and 11) and protein sample to be assessed was added to each buffer and kept for incubation at room temperature for 2 hours. Reading was taken in CD instrument from 190 nm to 260 nm at 25ºC for each pH where baseline measure was taken using 10mM phosphate buffer.

The effect of SDS on PNA was studied by using different concentration of SDS (0mM, 0.05mM, 0.1mM, 0.5mM, 1mM, 2mM, 5mM, and 10mM) prepared from 10mM and 20mM stock to which protein was added. Samples were prepared using different concentration of SDS, buffer and protein sample which was incubated in room temperature for 2 hours. The sample measurement was carried out using CD from 190-260 nm at 25ºC. Similarly, the

effect of GdnHCl on PNA was studied by using different concentration of GdnHCl (0M, 0.1M, 0.25M, 0.5M, 1M, 1.5M, 2M, 2.5M, 3M, 4M) prepared from 8M stock to which protein sample was added and incubated for 2 hours followed by CD measurements from 190-260 nm at 25ºC.

4.5 THERMAL PROFILING

CD spectroscopy can also be used to monitor changes of secondary structure within a sample over time with respect to temperature. CD instrument equipped with temperature control units was used to heat the sample in a controlled manner from 30ºC to 90ºC.A concentration of 0.1 mg/ml was taken for thermal denaturation study in CD. As the protein undergoes transition from folded to unfolded state, the CD spectrum at certain wavelength is monitored and plotted against temperature, yielding thermal denaturation curve which was used for stability analysis. Thermal denaturation was also assessed by UV-VIS spectrophotometer at 280nm from 30ºC to 90ºC assisted with peltier and graph was plotted indicating Absorbance versus temperature.

5. RESULTS AND DISCUSSION

5.1 PURIFICATION AND CHARACTERIZATION

In the past few years lectins have gained attention due to their ability to agglutinate human thymocytes, peripheral blood lymphocytes, and peripheral blood cells of various types of leukemia(27).This lead to purification of many plants lectins and its characterization. We successfully isolated and purified from peanut seeds *(Arachis hypogea)* by size exclusion chromatography (SEC). Pure fractions of PNA were obtained after salting out with 90% ammonium sulfate precipitation followed by size exclusion chromatography. Purity of this protein was checked using SDS-PAGE(28), where two prominent bands corresponding to 28 KDa and 16 KDa were observed. 28 KDa band indicated that under denaturing condition the subunits of PNA were separated, confirming that isolated PNA was a homotetrameric protein of MW 110 KDa (Fig5). The other band obtained at 16 KDa may be because of unspecific cleavage of either subunit. The concentration of the protein was then estimated by Bradford assay(26) and was found to be 0.5mg/ml.

Legume lectins including PNA have been previously shown to be composed of primarily β-sheet structures forming jelly roll motif (29).To further confirm the secondary structural

features present in the purified PNA we did FT-IR analysis. The FT-IR spectrum showed peaks at 1550 cm^{-1} and 1750 cm^{-1} (Fig6a) that corresponds to the β-sheet structures present in the proteins (30).To further confirm the secondary structure we performed Circular Dichroism spectroscopy that revealed and main peak was found around 223 nm (Fig6b) which normally depicts α-helical structures but is typical of β-sheet present specifically in lectins(31). Thus, from FTIR and CD studies of all the fractions of elute, it was concluded that fraction 4 to 7 was relatively very pure in comparison to other fraction collected from SEC. The further experiment for the characterization of PNA was carried out using these four elutes.

For Unfolding studies on PNA, CD spectropolarimeter and extrinsic fluorescence using ANS were employed. Denaturation was performed under using three chaotropic agents which include: i) pH, ii) SDS concentration, and iii) GdnHCl and the changes were monitored.

5.2 EFFECT OF pH

ANS shows a characteristic blue shift from 530 to 470 nm when its binds to hydrophobic patches of proteins mostly accompanied by an increase in the fluorescence intensity and is mostly used to study the molten globule states in proteins(32).When increasing pH from 2 to 11,maximum fluorescence intensity was shown at pH 2 where as from 3 to 11,the intensity change was not significant (Fig7a). The presence of the dimeric form of peanut agglutinin at a pH below 2.5 (33) may be an explanation for such high fluorescence as more hydrophobic groups are exposed in the dimeric form in comparison to the tetrameric form. From pH i.e.5 to 11,as the fluorescence was almost constant depicting that the protein was stable within this range or the structural changes that occurred could not be depicted by ANS alone.

As for the CD studies with varying pH from 2-11, there was a characteristic shift from 223 nm to 208 nm (Fig8a)., a typical of α-helix in proteins(34) at extreme pH of 2,9 and 11.We can thus infer that at very low or very high pH, ionizable groups in protein structure can get disrupted. This can result in altered electrostatic interactions between amino acids and modification of the protein structure. (35)

5.2 EFFECT OF SDS

Ionic detergents like SDS can denature proteins by strong binding to charged and hydrophobic side chains even at millimolar concentration(12).This property of SDS was employed to study conformational changes of PNA with varying concentrations of SDS. Increase in SDS concentration from 0mM to 0.05mM show decreased fluorescence and from SDS concentrations 0.5mM to 2mM intensity change was not significant. Maximum fluorescence was found at 10mM (Fig9a) which may be due to exposure of hydrophobic patches.

CD studies indicated a significant native peak at 223nm with no SDS concentration till 0.5Mm, indicating β-sheet conformation. Increasing concentration of SDS from 1M to 10M shows a characteristic shift from 223nm to 208nm a typical feature of α-helix. From this we can infer that at high SDS concentration the interaction between amino acid may have been altered resulting in modification of protein conformation (Fig10a).

5.3 EFFECT OF GUANIDINE HYDROCHLORIDE

Following the denaturation of PNA by GdnHCl and subsequent ANS fluorescence measurements, we observed that initially there is an increase in the ANS fluorescence intensity pointing towards the exposure of the hydrophobic clusters within the tetrameric protein. Small concentration of Guanidium hydrochloride may be able to deoligomerize the tetrameric protein exposing its hydrophobic clusters. High fluorescence could also be due to the formation of a molten-globule state, an intermediate formed during the process of folding and unfolding of proteins(36).Maximum fluorescence was found between 1-1.5 M of guanidine hydrochloride after which the fluorescence drastically reduced and the minimum was observed at 4M GdnHCl indicating complete unfolding (Fig 11a).

As for CD studies indicated native peak at 223 nm at no GdnHCl concentration till 1.5 M GdnHCl which points to denaturation, more specifically deoligomerization where each monomer still retains β-sheet conformation like that of its tetrameric parent. Higher concentration than of 1.5 M there is shift the CD spectrum towards lower wavelengths (208

nm) and reduction in the ellipticity indicating complete denaturation and formation of random coiled states (Fig12a).

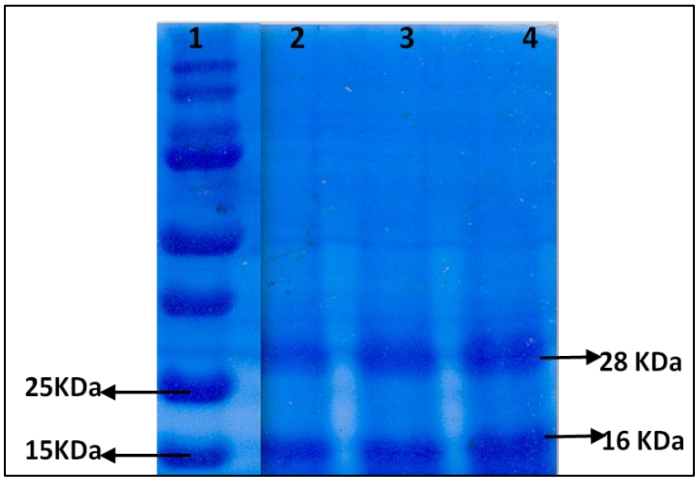

Fig5. Coomassie blue stained 10% SDS –PAGE gel. The Denaturing gel comprises of two prominent bands one at 28KDa and other at 16KDa (LANE 1: Marker protein, LANE 2, 3, 4: PNA)

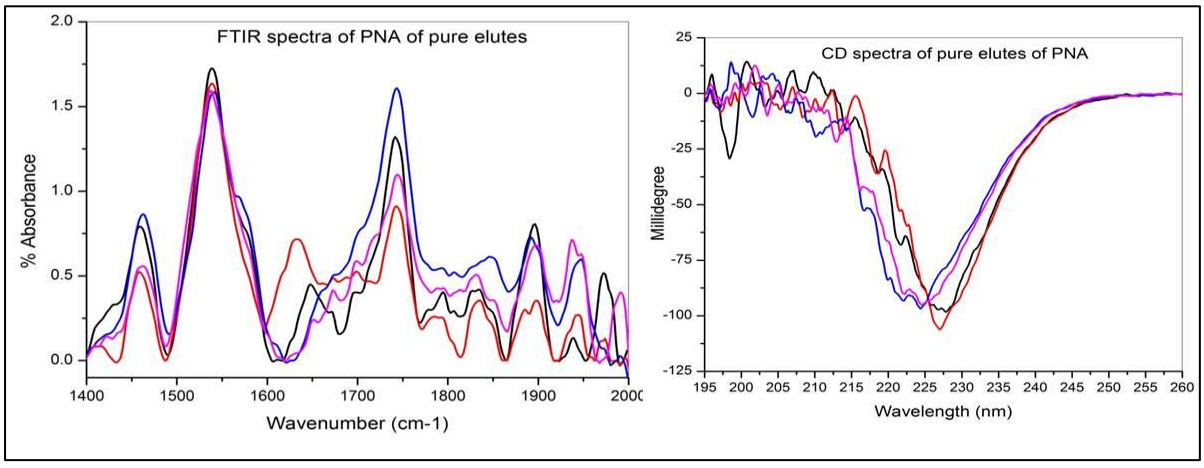

Fig6a. FTIR spectra of intact PNA

Fig6b. CD spectra of intact PNA

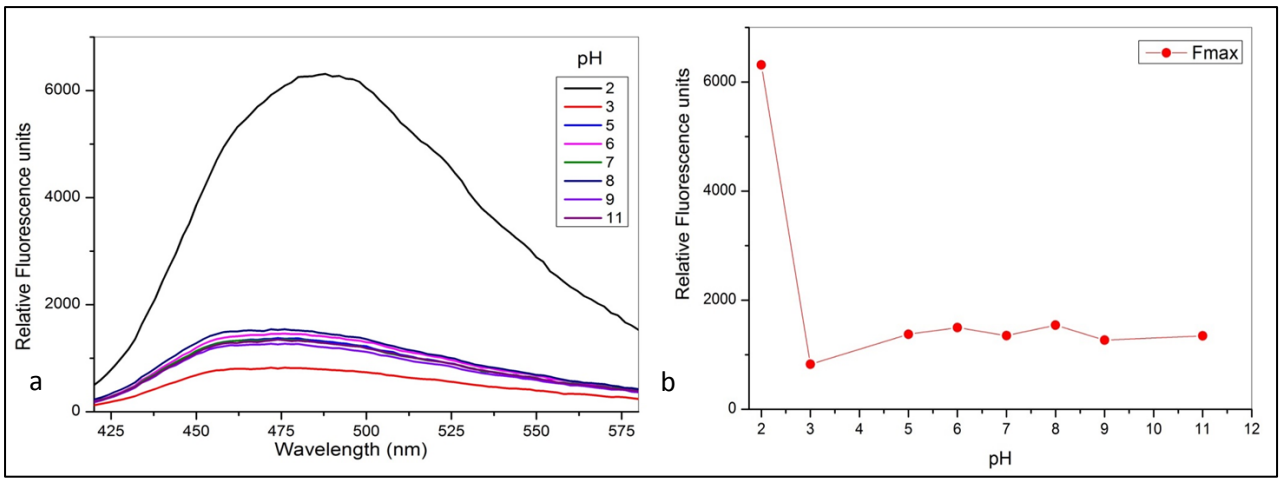

Fig7a. ANS fluorescence of PNA at different pH, Fig7b. Fmax at 470 nm at different pH

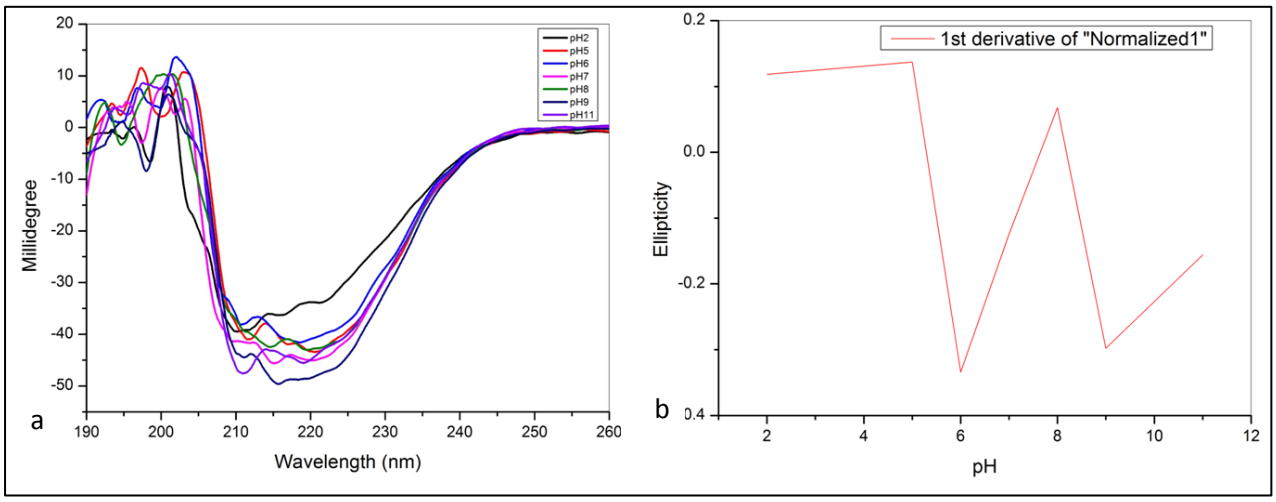

Fig8a. CD spectra of PNA at different pH

Fig8b. 1st derivative spectra of ellipticity at 208nm for different pH.

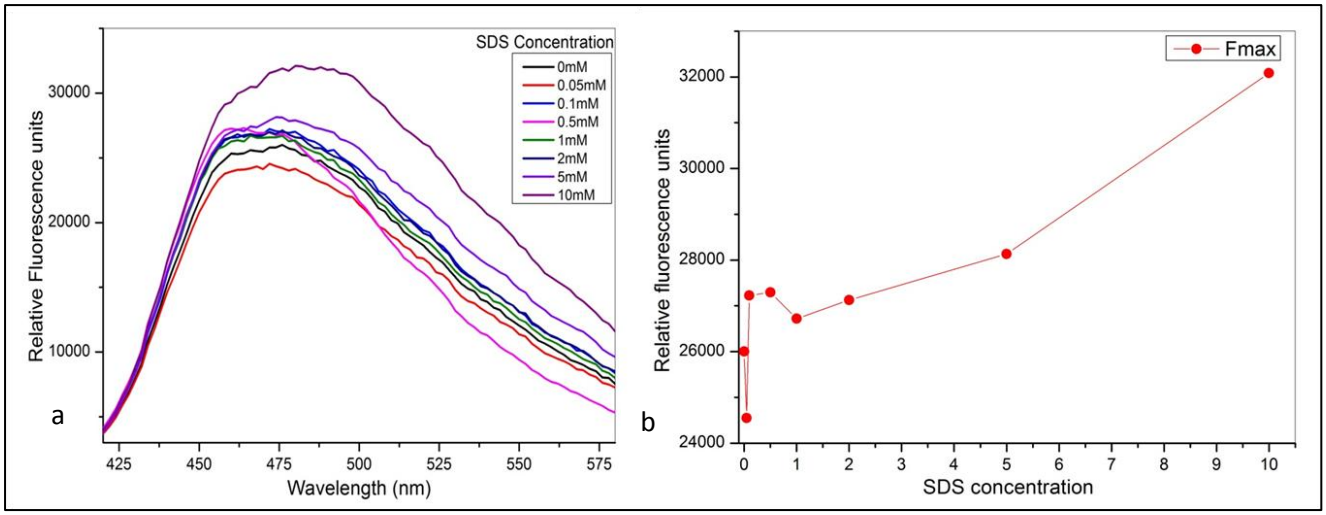

Fig9a. ANS fluorescence of PNA at different SDS conc. , Fig9b. Fmax at 470nm at different SDS concentration

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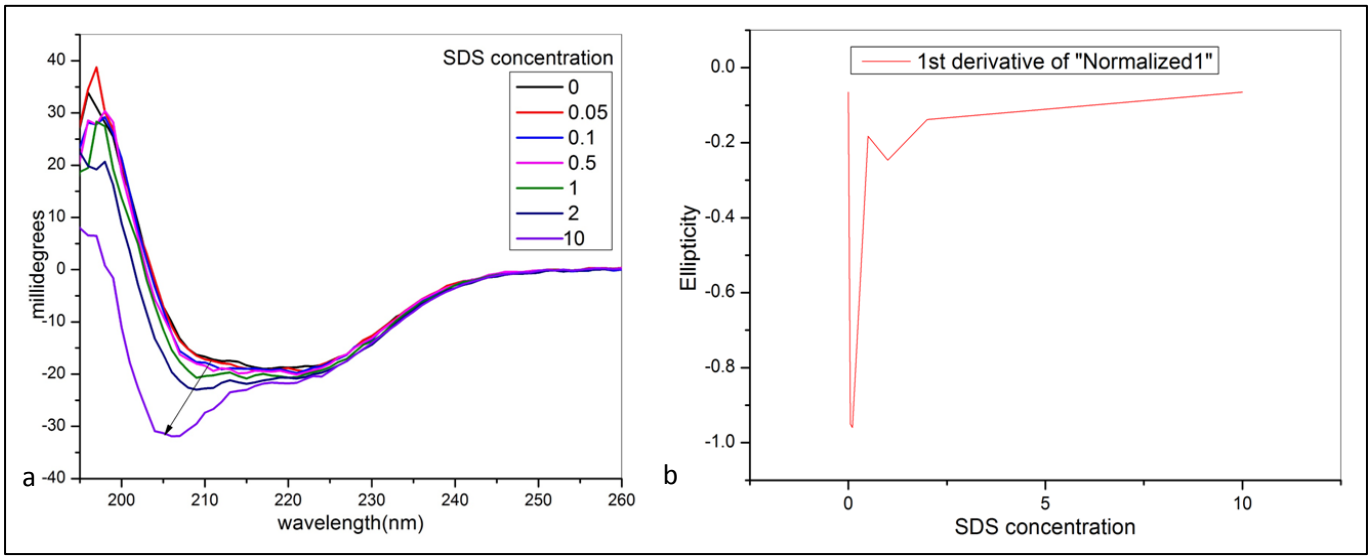

Fig10a. CD spectra of PNA at different SDS concentration.

Fig10b.1st derivative spectra of ellipticity at 208nm for different SDS concentration.

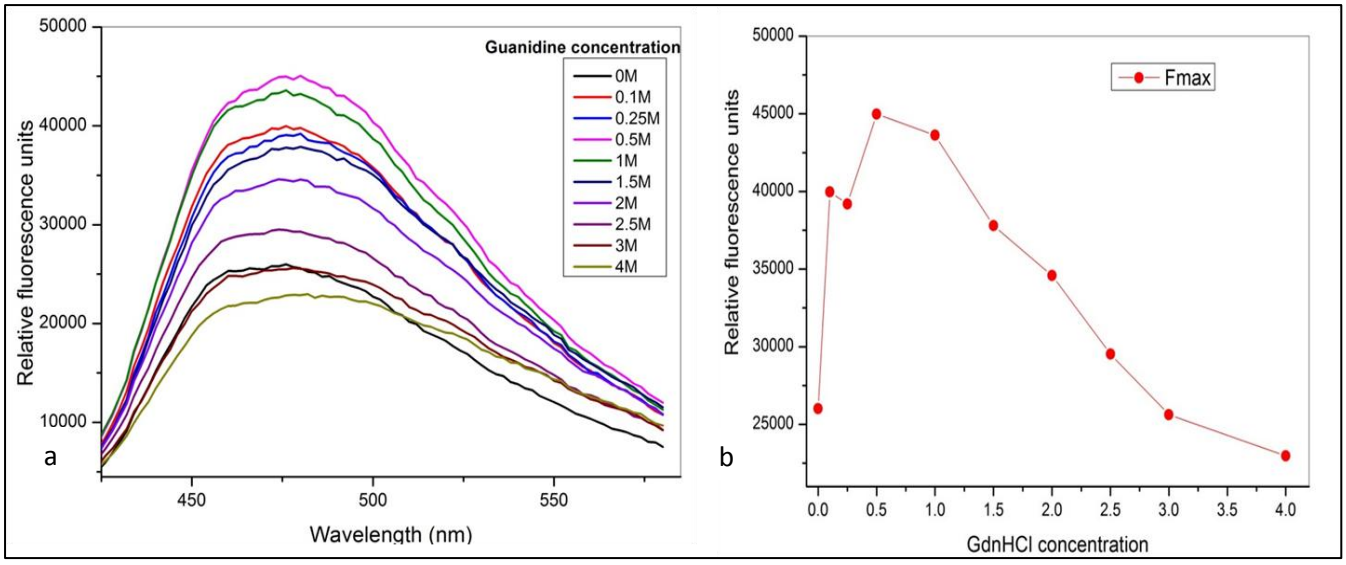

Fig11a. ANS fluorescence of PNA at different, concentration of GdnHCl.

Fig11b. Fmax at 470nm at different concentration of GdnHCl.

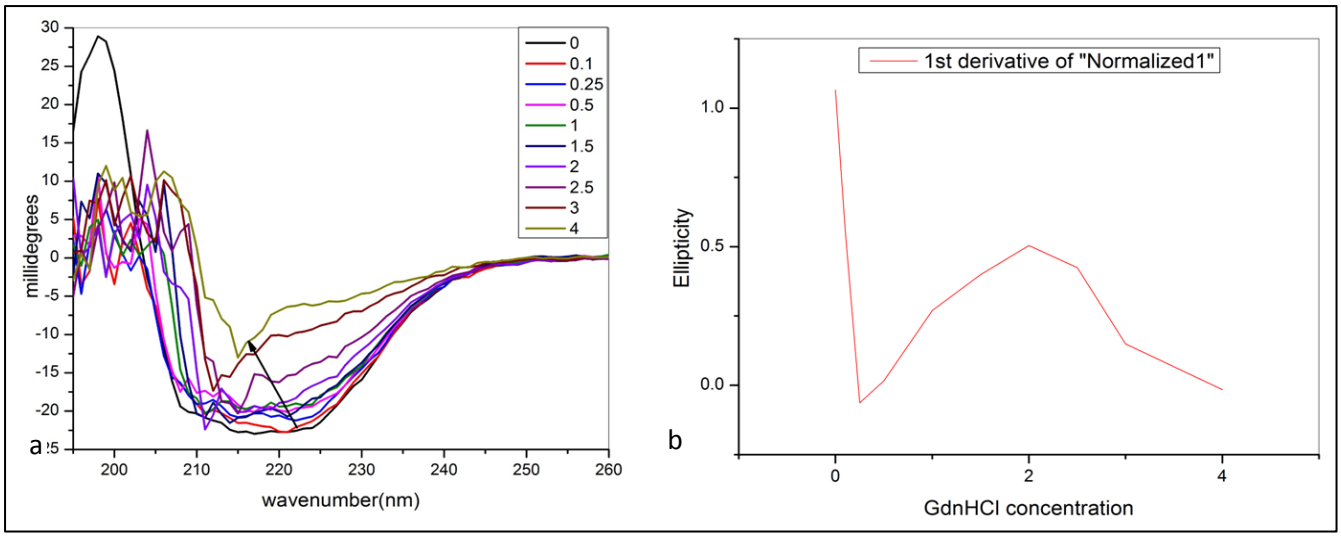

Fig12a. CD spectra of PNA at varying concentration of GdnHCl.

Fig12b. 1ST derivative spectra of ellipticity at at 208nm for different GdnHCl concentration.

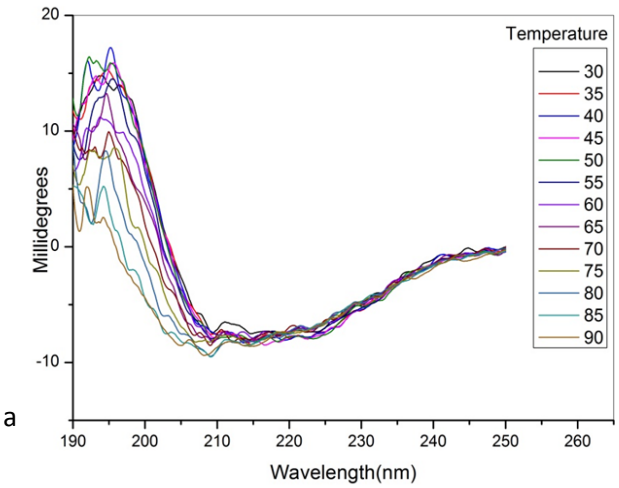

Fig13a.CD spectra of PNA at different temperatures.

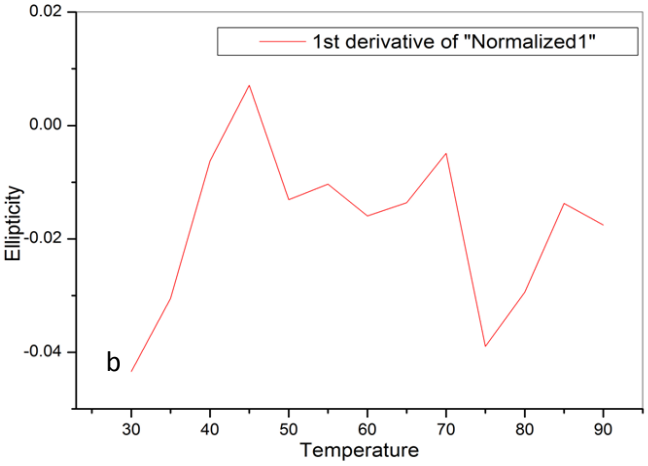

Fig13b. 1ST derivative spectra of ellipticity at 208nm for different temperature.

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